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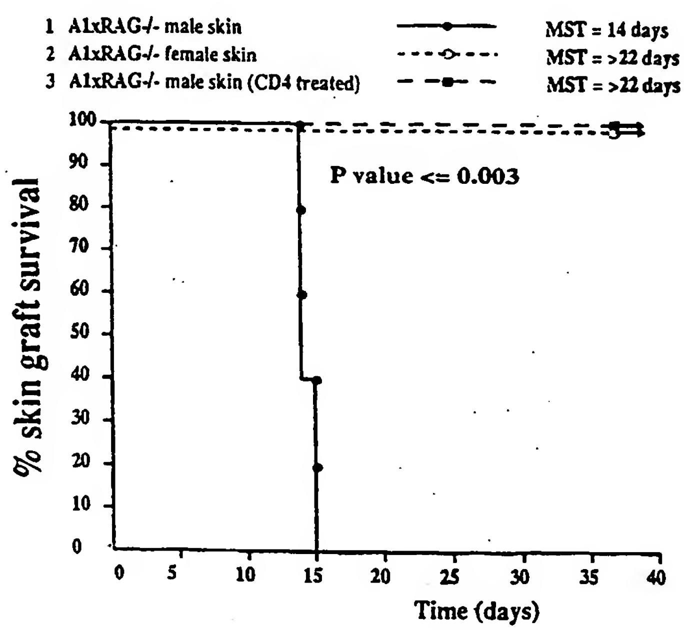
(57) Abstract

The invention provides a genetically modified non–human mammal having a population of CD4 positive T cells specific for one or a limited number of selected antigens, including at least one transplantation antigen capable of rejecting a tissue transplant containing the transplantation antigen and if applicable the other selected antigens. A genetically modified animal according to the invention has T cell receptor genes which encode a T cell receptor specific for the transplantation antigen. The genetically modified mammal is useful in studying immunological tolerance, in particular in the mechanisms of tolerance to and the rejection of tissue grafts, and in pregnancy. The animals are also useful for testing agents for biological activity in promoting or reducing immunological tolerance.



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TRANSGENIC MODEL COMPRISING TCR ALPHA AND BETA CHAINS

This invention relates to a genetically modified non-human mammal having limited T cell receptors, capable of mounting an immune response to only one or to only a limited number of antigens, and of being tolerised to the antigen or antigens. The invention also relates to methods of testing biologically active compounds using such a genetically modified mammal.

Immunological tolerance is a state of unresponsiveness of the immune system which is specific for a particular antigen. An important aspect of tolerance is self-tolerance, which is an unreactiveness to self that allows the body to distinguish between self antigens presented to it on normal tissues and potentially dangerous situations such as infection. It is a generally held view that the immune system is unreactive to self antigens, but maintains a broad repertoire of receptors sufficient to recognise any non-self antigen presented to it (central tolerance). It is also generally accepted that there must be some fail-safe mechanisms that operate to minimise the effects of any mistakes that may lead to autoreactivity (peripheral tolerance). The possible mechanisms for tolerance, in particular for peripheral tolerance, are reviewed in Cobbold et al. 1996 Immunol. Rev. 149:5-33.

Tolerance is clearly a matter of practical importance in treatments which require the introduction of foreign antigens into the body, such as tissue transplantation and vaccination, and in diseases which are characterised by immunosuppression such as AIDS or by a breakdown in self-tolerance resulting in autoimmunity. However, the techniques for studying tolerance and for investigating the effects of pharmaceutical compounds on tolerance have until now been limited.

Until relatively recently, neonatal tolerance induction was the primary technique for experimental study of tolerance. Specific tolerance to grafted skin was induced in mice by neonatal injection of spleen cells from a different strain. The resulting mice showed tolerance to skin grafts from the donor strain. Moving on from the neonatal induction models, transgenic methods for introducing foreign transgenes into mice have allowed the study of specific antigens in a defined genetic background which are treated as self by the immune system.

Techniques for inducing tolerance in the mature adult immune system have also been developed. Monoclonal antibodies against the murine CD4 and CD8 antigens are a powerful means to produce tolerance to a variety of different antigens in the adult mouse. Depletion of the CD4 or CD8 T cells is not necessary; a blockading with either $(Fab^1)_2$ fragments or a non-depleting isotype of anti-CD4 or anti-CD8 antibody is especially effective. Combinations of CD4 as well as CD8 antibodies can be used for the extended periods found necessary for inducing tolerance in some of the more difficult skin graft systems (Cobbold et al, 1990 Eur. J. Immunol. 20:2747-2755).

The discovery that tolerance could be induced to transplanted organs under the cover of non-depleting CD4 and CD8 antibodies given together was a major breakthrough (e.g. Cobbold et al. 1990). Because skin grafts can be used as both the tolerogen and later test challenge, the system gives a consistent read-out for rejection/tolerance *in vivo* that does not depend on making assumptions about the effector cells or the antigens, as is necessary for *in vitro* measurements. The main advantage of this approach over that of neonatal tolerance induction, or the introduction of foreign transgenes, is that it makes it possible to focus on events that take place without the thymus (by removing it) and without other previous exposure to the antigen during the development of the immune system. This allows more precise control of the timing of

tolerance induction and antigen challenge. There is also a wider choice of how the antigen is presented for tolerance by using different organs as grafts.

However, in all of the techniques described, there is a limited amount of specific information that can be gained relating to the mechanisms for rejection and tolerance, because of the complexity of the immune system and the immune response.

It is therefore an object of the invention to provide a simplified system for observing an immune response and/or tolerance to a particular antigen or to a limited number of antigens.

It is another object of the invention to provide a system for testing the effects of treatments on an immune response and/or tolerance to a particular antigen or to a limited number of antigens.

Surprisingly, it has been found that mice having only CD4 positive T cells, all of which have a single T cell receptor specific for the same transplantation antigen, can reject skin grafts. Previously, it had been thought that the role of CD4 positive T cell in graft rejection was mainly to provide T cell help to either CD8 positive T cells to become cytotoxic, or to B cells to make antibody. Thus, CD8 positive T cell receptor transgenic mice have been made (Hammerling et al. 1993 *Immunol. Rev.* 133:93-104). Furthermore, it was not expected that such a CD4 T cell receptor "monoclonal" mouse once it had been found to reject grafts, could be tolerised to the transplantation antigen. Previously, it was often assumed that tolerance works either by eliminating the specific T cells (clonal deletion) or by inducing a separate population of suppressor cells. In fact, tolerance can be effectively induced by treatment with non-depleting anti-CD4 antibody.

The invention provides in one aspect a genetically modified non-human mammal having a population of CD4 positive T cells specific for one or a limited number of selected antigens, including at least one

transplantation antigen, the mammal being capable of an immune response against the transplantation antigen and of being tolerised to the transplantation antigen.

Preferably, the genetically modified animal according to the invention is capable of rejecting a tissue transplant containing the transplantation antigen and if applicable the other selected antigens.

A genetically modified animal according to the invention has T cell receptor genes which encode a T cell receptor specific for the transplantation antigen. The T cell receptor genes will generally be stably integrated into its genome. Most T cell receptors are made up of α and β chains which are encoded by separate genes. The T cell receptor genes employed in the invention may be derived for example from cloned α and β chain genes for T cell receptors specific for the transplantation antigen, or from α and β chain genes prepared synthetically using sequence information from cloned genes. In another alternative, the genes are derived from α and β chain genes for T cell receptors which are not necessarily specific for the transplantation antigen. In that case, well known methods such as site-directed mutagenesis may be used to alter the gene sequences to achieve transplantation antigen specificity.

Preferably, the genetically modified animal according to the invention lacks a normal population of CD8 positive T cells, or B cells, or both. More preferably, the animal has no functional CD8 positive T cells or B cells. An absence of lymphocytes other than CD4 positive T cells provides a far simpler system for studying the activity of the immune system in relation to a selected transplantation antigen.

An absence of CD8 positive T cells and B cells may be as a result of the animal having a deficiency in lymphocyte receptor recombination such that no CD8 T cell receptors or B cell receptors (antibody) are expressed. For example, mice which are deficient in RAG-1 and/or RAG-2 (recombinase activating gene no. 1 and/or no. 2) activity

have an inability to initiate VDJ recombination in the lymphocyte receptor gene and therefore cannot generate mature lymphocytes. RAG mutations can therefore be used in the invention to eliminate functional lymphocytes other than the specific CD4 lymphocytes directed to the transplantation antigen (and specific CD4 lymphocytes directed to any other selected antigens). RAG-1$^{-/-}$ mice are described in Alt et al. 1992 Ann. N. Y. Acad. Sci. 651:277-94 and Mombaerts et al. 1992 Cell 68(5):869-77.

The population of CD4 positive T cells which are specific for the transplantation antigen (or if appropriate, other selected antigens), will generally form the majority of the entire CD4 positive T cell population of the animal, for example 50-60% or more of the CD4 positive T cells. In the case of a recombination deficiency such as a RAG-deficiency, all functional CD4 positive T cells will be specific for the transplantation antigen (or other selected antigens).

The transplantation antigen to which the CD4 positive T cells are directed may be any transplantation antigen which is not expressed in the animal itself, or not expressed in the animal in a form recognisable by the CD4 positive T cells. A transplantation antigen is defined as a tissue antigen which can be introduced into the body in a tissue transplant in such a manner that it is recognised by the immune system. A tissue antigen is an antigen found on body tissues and organs. Tissue transplants for the purposes of the invention include foetuses in the maternal environment.

In a particular species there are characteristic transplantation antigens, each of which may be present or absent in any given individual, or any given group of individuals such as a strain, of the species. A transplantation antigen may occur in a variety of different forms such that it is recognisable as being foreign between different individuals or between different groups of individuals in the species. Transplantation antigens include major transplantation antigens such as the MHC (major histocompatability complex) antigens, or minor transplantation antigens

such as for example the murine male transplantation antigen H-Y, the murine β_2 microglobulin antigen H-1, or mitochondrial antigens.

For certain transplantation antigens, in particular some of the minor transplantation antigens, CD4 positive T cells specific for a single antigen may not be sufficient for rejection of a tissue graft. In the case of these minor transplantation antigens, further CD4 positive T cells having specificity for one or a few further antigens are provided, for example by stably integrating further specific T cell receptor (TCR) genes into the animal. The murine male transplantation antigen H-Y is an example of a minor transplantation antigen which, conveniently, is sufficient on its own for graft rejection in an animal which has only H-Y antigen specific TCRs.

In another aspect, the invention provides a method of screening for biologically active compounds, which method comprises administering the compounds to a genetically modified non-human mammal as described herein and observing the effect of the compounds on the ability of the mammal to reject or maintain a transplant containing the transplantation antigen.

In the case of a simple male/female transplantation antigen such as the male H-Y antigen, the method according to the invention can employ a female recipient mouse and graft tissue from a congenic male donor mouse. Where a male or female transplantation antigen is not used, the recipient and donor animals are preferably from congenic strains with the donor strain being transgenic for the transplantation antigen. This means that the system is kept as simple as possible with the only antigenic difference between the donor and recipient being the transplantation antigen (and other selected antigens if applicable).

The invention enables any specific changes in the T cell population, such as activation markers or cytokine production, to be easily monitored by conventional immunological techniques during transplantation or during tolerance induction or maintenance, and also

during and after pregnancy. A so-called "Toleramouse" is thus an ideal tool for investigating the mechanisms of immunosuppression and tolerance induction. Previously, such experiments had to be performed either in normal inbred or congenic mice in which the frequency of T cells against any single antigen is too low for direct observation using simple immunological techniques such as immunofluorescent staining or cytokine staining assays.

The study of tolerance mechanisms in pregnancy may be performed on pregnant animals, in particular pregnant animals as described herein near to and after the end of pregnancy term. Particularly useful are pregnant females carrying one or more male foetuses displaying a male transplantation antigen, where the pregnant female has TCRs directed against the male transplantation antigen. Pregnant animals carrying male and female foetuses can be compared. The studies performed usefully include looking at the T cell population and T cell markers, including cytokine profiles.

It is envisaged that the invention will be particularly useful for testing potential or existing therapeutic agents:

i) for wanted or unwanted tolerogenic or immunosuppressive (side) effects;

ii) for interference with the tolerance process, either once it has been established or during the induction of tolerance by appropriate therapeutic agents such as CD4 monoclonal antibody.

More specifically, the invention may be used to screen for agents which:

i) have tolerising effects in a similar manner to CD4 monoclonal antibodies;

ii) do not interact with tolerance-inducing drugs;

iii) enhance the immune response in diseases which feature immunosuppression such as AIDS and cancer;

iv) interact in desired ways with other drugs or with vaccines.

The invention is now further described in the following examples, which are not intended to limit the scope of the invention in any way.

EXAMPLES

Example 1 - Construction of Transgenic Mice

Methods

Mice:

CBA/Ca (Harlan/Olac, UK) mice were bred under specific pathogen free conditions and all experimental mice were maintained in the animal facility of the Sir William Dunn School of Pathology, Oxford, in a filtered cage system (Maximiser, Thorens Caging Inc., Hazelton PA, USA). RAG-$1^{-/-}$ mice that had been bred onto an H-2^k background were obtained from Dr. B. Stockinger (NIMR, London, UK).

Generation of A1(M) transgenic mice:

To generate transgenic mice we used the TCR α and β chain from the A1 CD4+ T cell clone isolated from CBA/Ca mice (Tonomari 1985. *Cell Immunol.* 96: 147-62). The A1 clone recognizes the minor histocompatibility antigen H-Y, present in male but absent in female mice, in the context of H2-E^k. The TCR expression was identified using primers specific for the Vα and Vβ gene families (Casanova et al. 1991. *J. Exp. Med.* 174(6): 1371-83), cloned and sequenced to check for productive rearrangement. The α chain was found to be encoded by LVα10-Jα30-Cα (EMBL accession no. AJ000157) and the β chain is encoded by LVβ5.1-Vβ8.2-Dβ2-Jβ2.3-Cβ2 (EMBL accession no. AJ000158) (Figure 1). EcoRI-EcoRI fragments containing the productively rearranged α or β chains were generated by RT-PCR. The oligonucleotides used for the amplification of the α chain were :

GCGAATCACAAGCACCATGAAGAGGCTG [SEQ ID NO: 1] and GCGAATTCCAGAC CTCAACTGGACCACAG [SEQ ID NO: 2]. The oligonucleotides used for the amplification of the β chain were: GCGAATTCAGAGGAAGCATGTCTAACACT [SEQ ID NO: 3] and GCGAATTCAGGATGCATAAAAGT TTGTCTCAGG [SEQ ID NO: 4].. The full length cDNAs were cloned into the human CD2 minigene (VA) in pBluescript. SalI-XbaI fragments were used for microinjections into CBA/Ca oocytes. Transgenic A1(M) founders were maintained on the CBA/Ca background and bred to homozygosity. Southern blot analysis indicated that the A1(M) line carries a single copy per haplotype of each of the transgenic Vα and Vβ chains.

Skin grafting

Pieces of tail skin approx. $0.5cm^2$ were grafted onto the lateral thoracic wall of anaesthetised recipient mice as previously described (Cobbold and Waldmann 1986. *Transplantation* 41: 634-639; and Qin et al. 1990. *Eur. J. Immunol.* 20: 2737-2745). Where two grafts were given simultaneously these were placed side by side in the same prepared graft bed. Plaster casts were removed on day 7 and the grafts observed daily, with rejection being defined as the day when no viable graft tissue could be seen. Statistical significance was determined using the LogRank method (Peto et al. 1977. *Br. J. Cancer* 35:1-39). All procedures were carried out in accordance with the UK Home Office Animals (Scientific Procedures) Act of 1986.

Immunofluorescent analysis and antibodies

Thymus, spleen or lymph nodes were removed, and erythrocytes lysed by isotonic shock. Cells were labelled in phosphate buffered saline (PBS) containing 0.1% (w/v) NaN_3, 1% (w/v) bovine serum albumin (BSA), and 5% (v/v) heat inactivated normal rabbit serum (HINRS:

to block Fc receptors) at 4°C. Antibodies used were: CD3ε (KT3-FITC), Vβ8 (KJ16-FITC), Vβ8.2 (F23.2-FITC), CD4-PE (Sigma P2942), CD8α-QR (Sigma R3762), B220-QR (Sigma R4262), CD25 (PC61-biotin), CD44-QR (Sigma R5638), SA-APC (Pharmingen 13049A), and FITC goat anti-mouse IgG (Sigma F0257). After labelling and washing, cells were fixed in 1% formalin and stored in the dark at 4°C. Four colour analysis was performed using a FACSort (Becton Dickinson, Oxford, UK) with dual laser (488nm and 633nm) excitation together with data aquisition and cross-beam colour compensation using CellQuest 3.1 software. At least 50,000 events were stored in list mode for further analysis and gating on forward and side scatters.

Intracytoplasmic cytokine staining was performed using spleen cells given a 2 hr stimulation in vitro with 50 ng/ml PMA (Sigma, P8139) + 500 ng/ml ionomicin (Sigma I0634) in phenol red free RPMI 1640 medium + 10% fetal calf serum at 37°C) with the addition of 10ng/ml Brefeldin A (Sigma, B7651) for a further 2 hr (Ferrick et al. 1995. *Nature 373*: 255-257). After washing, cells were fixed in 2% v/v formaldehyde in PBS (20min, 4°C), washed, and permeabilized with PBS + 0.5% saponin (Sigma S-2149). The following antibody conjugates were added in saponin buffer for 30 mins at 4°C, followed by extensive washing in saponin buffer followed by PBS + 0.1% azide + 1% BSA, + 5% HINRS: anti-IL-2 (S4B6-FITC; Pharmingen 18004A), anti-IL4 (11B11-FITC; Pharmingen 18194A), anti-IFN-γ (XMG1.2-FITC; Pharmingen 18114A). Cells were finally labelled with antibodies to surface CD4 and CD44, fixed in 1% formalin, and analysed on a FACSort as above. The conditions of stimulation, staining and analysis were such that normal CBA/Ca $CD4^+$ spleen cells were essentially negative for all cytokine stains.

Treatment with CD4 monoclonal antibody

The non-depleting rat IgG2a anti-mouse CD4 (YTS 177.9 Qin et al. 1990) was made by growing the hybridoma in a hollow fibre bioreactor, and was purified under sterile and low endotoxin conditions by precipitation with 50% saturated ammonium sulfate. (These are standard techniques known to a person skilled in the art. An example of the technique is also available on http://www.molbiol.ox.ac.uk/www/pathology/tig/mprod.html). Grafted A1(M)xRAG$^{-/-}$ mice were given 5 x 1mg intraperitoneally from the day of grafting over a two week period.

Results and Discussion

Analysis of A1(M) mice transgenic for TCR against H-Y+H2-E^k.

Thymus, spleens, and lymph nodes from A1(M) mice were analysed by 3 colour immunofluorescence to determine whether the expression of the transgenic TCR led to the predicted functional modification of the T cell repertoire. The thymi of female A1(M) mice were found to have a strong bias towards the generation of CD4$^+$CD8$^-$ rather than CD8$^+$CD4$^-$ T cells, as expected from an increased positive selection of the MHC-II restricted anti-H-Y TCR. This led to a CD4/CD8 ratio in the peripheral lymphoid organs in excess of 10:1, and expression of the Vβ8.2 transgenic receptor on more than 90% of CD3$^+$ cells. In contrast, male A1(M) mice had smaller thymi (a mean of 6x10^6 total thymocytes compared to 8x10^7 in females), with a mature CD4/CD8 ratio close to 1:1, and a similar expression of Vβ8.2 to non-transgenic CBA/Ca mice, suggesting clonal deletion of anti-H-Y transgenic T cells and escape of endogenous TCR rearrangements. These A1(M) mice were then crossed onto a RAG-1$^{-/-}$ background, to eliminate all B cells and T cells expressing other TCR molecules encoded by endogenous TCR rearrangements, so that any

ability of H-Y specific $CD4^+$ T cells to reject male skin grafts could be unambiguously identified.

Positive selection in female and negative selection in male A1(M)xRAG-$1^{-/-}$ mice

Immunofluorescent staining of A1(M)xRAG$^{-/-}$ mice confirmed that the anti-H-Y TCR was functional, as positive selection and the generation of $CD3^+CD4^+CD8^-$ thymocytes was only observed in female thymi, while male thymi were much smaller, with very few $CD4^+CD8^-$ cells. When we looked in more detail at these few $CD4^+CD8^-$ cells we found them present in similar numbers in both male A1xRAG-$1^{-/-}$ and RAG-$1^{-/-}$ controls, and that none of them expressed CD3 but were mostly $CD11c^+$, suggesting they may be related to $CD4^+$ immature dendritic cells (Winkel et al. 1994 *Immunol. Lett.* 40: 93-9) rather than T cells that had somehow escaped deletion. Staining of lymph-nodes confirmed that only $CD4^+$ and not $CD8^+$ T cells were present in female A1(M)xRAG-$1^{-/-}$, and that clonal deletion in the male reduced the number of $CD4^+$ cells down to that seen in RAG-$1^{-/-}$ mice (and these were again $CD3^-CD11c^+$). The expression of the TCR in female A1(M)xRAG-$1^{-/-}$, as measured by CD3 or Vβ8.2 staining, was lower than in a normal CBA/Ca mouse (approx. 30% of the median fluorescence level), but similar to that of the A1(M) founders, which may be a property of the CD2 expression system (G. Stockinger, personal communication).

Rejection of male skin by female A1(M)xRAG$^{-/-}$ mice

In initial experiments in two laboratories, a total of 8 female A1(M)xRAG-$1^{-/-}$ mice were given single male skin grafts, four of which were rapidly rejected (within 16 days) and a further two were eventually rejected in a chronic fashion. Subsequently, a group of 5 female A1(M)xRAG-$1^{-/-}$ mice were simultaneously grafted with male and female CBA/Ca skin in the same graft bed. All of the male grafts were rapidly rejected (within 14

days), while the female grafts remained perfectly healthy (Figure 2). A second group of five was grafted in an identical fashion but were also treated with saturating amounts of a monoclonal antibody that blocks CD4 function *in vivo*. All these grafts were accepted, proving that the rejection was both CD4 dependent and male specific. As illustrated in Table 1, non-depleting CD4 monoclonal antibodies produce an indefinite tolerant state (ability to accept fresh male skin grafts) even well after the antibodies are no longer in circulation.

Mechanism of CD4 dependent graft rejection

The A1(M)xRAG-$1^{-/-}$ mice should have no $CD8^+$ T cells or antibody producing B cells that might be able to act as effectors of graft rejection, and this was checked by staining spleen cells from two female A1(M)xRAG-$1^{-/-}$ mice that had been allowed to reject two sequential male grafts, and had been grafted with a third male skin 7 days previously, such that if there was any hypothetical expansion of, for example, a novel $CD8^+$ population during graft rejection, this should become visible. However, it was confirmed that there was no $CD3^+CD8^+$ staining above background (Figure 3), and that CD25 expression was limited to the $CD3^+CD4^+$ subset. Even if there had been some $CD8^+TCR^+$ cells that remained below the level of detection, these should have been unable to interact effectively with the H-$2E^k$ presented male antigen, and would thus be extremely unlikely to contribute to graft rejection. Similarly, there were no surface Ig^+ B cells that might have been able to contribute an antibody response. There was clear evidence that the male graft was indeed being recognized by the transgenic anti-male TCR on $CD4^+$ T cells, as the majority (approx. 70%) of these could be shown to be recently memory or recently activated cells (expressing CD44) as well as producing both IFN-γ, and to a lesser extent, IL-4 (Figure 3). Interestingly, all T cells (both $CD44^+$ and $CD44^-$) appeared to be expressing IL-2 by this method of intracytoplasmic staining.

It is therefore clear that the transgenic $TCR^{+}CD4^{+}$ T cells in A1(M)xRAG-$1^{-/-}$ females are sufficient to reject male skin. The mechanism by which the animals are made tolerant by non-depleting CD4 antibodies is neither deletion nor a conversion of Th1 to Th2 cells. The tolerisation may be due to a direct, $CD4^{+}$ T cell mediated cytotoxicity or it may be due to an indirect, perhaps cytokine mediated, help for macrophages or another antigen non-specific effector cell. Recent data suggesting that neither Fas/FasL nor perforin (Selvaggi et al. 1996 *Transplantation.* 62(12): 1912-5) are required for CD4 mediated rejection of MHC-I disparate skin would tend to favour the latter hypothesis. The MHC-I restricted $CD8^{+}$ arm of the immune response in normal mice would therefore seem to be mostly dependent on (Antoniou et al. 1996 *Eur. J. Immunol.* 26(5): 1094-1020), and an amplification of, the MHC-II restricted $CD4^{+}$ response that we have shown here can itself provide all the necessary T cell functionality for graft rejection.

<u>Example 2 – Testing of reagents for Immunosuppressive or Tolerogenic effects in A1(M) x RAG$^{-/-}$ mice</u>

A variety of different agents was tested. The testing methods used were as follows and results are given in Table 1.

a) Female A1xRAG-1-/- mice were given 10^7 male A1xRAG-1-/- bone marrow or spleen cells intravenously. They were then grafted with male tail skin after a further 6 weeks to test for tolerance, which was accepted indefinitely. Immunofluorescent analysis of peripheral blood and spleen cells indicated substantial deletion of the CD4+ T cells in the tolerant mice, but not in non tolerant controls that had been given female marrow or spleen cells.

b) Female A1xRAG-1-/- mice were given 5 x 1mg or a single injection of 1mg of non-depleting rat IgG2a anti mouse CD4 (YTS 177.9 Qin et al 1990 Eur. J Immunol 20: 2737-2745) at the time of grafting

male CBA/Ca tail skin. These grafts and subsequent male skin grafted at 42 and 84 days were accepted indefinitely. Analysis of blood, lymph-nodes and spleen cells from these mice demonstrated that CD4+ T cells had not been clonally deleted, and similar proportions of Th1 and Th2 cells could be identified by immunofluorescent staining for cytokines in both the tolerant mice and control animals that had not been treated with CD4 antibody but had received equivalent male skin grafts.

c) Female A1xRAG-1-/- mice were given 5 x 1mg of monoclonal antibody to CD25 (PC61: Osawa et al., Immunol. lett. 1989 20: 205-12) from the time of grafting with male CBA/Ca tail skin.

d) Female A1xRAG-1-/- mice were given 5 x 1mg of monoclonal antibody to CD40 ligand (MR1: Larsen et al. Transplantation 1996 61: 4-9) from the time of grafting with male CBA/Ca tail skin.

e) Female A1xRAG-1-/- mice were given 5 x 1mg of monoclonal antibody to CD28 (37.51: Sperling et al., J. Immunol. 1993 151: 6043-50) from the time of grafting with male CBA/Ca tail skin.

Table 1

Examples of testing reagents for immunosuppressive or tolerogenic effects in the Toleramouse

Agent tested	Source	No. Tested	Effect	Mechanism	Comments
Male antigen iv.	Bone marrow	6	Full tolerance to male skin	Predominantly clonal deletion	Model for blood transfusion effect
Male antigen iv.	Spleen cells	6	Full tolerance to male skin	Predominantly clonal deletion	Model for blood transfusion effect
Non-depleting CD4 monoclonal antibody	YTS 177.9	6	Full tolerance to male skin	Not deletion and not Th1 -> Th2	Accepted 2nd and 3rd male skin grafts
Anti-CD25 monoclonal antibody	PC61	6	Immuno-suppression	Not done	Rejection delayed
Anti-CD40 ligand monoclonal antibody	MR1	6	None	Not applicable	Dose regimen may not have been optimal
Anti-CD28 monoclonal antibody	37.51	3	None	Not applicable	Antibody preparation may not have been active

Example 3 - Construction of A1(M)xRAG-1$^{-/-}$ mouse from sequence information only

Making TCR transgenic mice is a routine procedure where the appropriate TCR alpha and beta chain encoding DNA is micro-injected into fertilized eggs from the strain of mice in which it is desired to express the TCR (for example the standard Olac CBA/Ca strain). The injected eggs are then transferred into foster mothers, and the offspring typed by standard methods (usually by PCR typing of tail DNA) to select those which have stably incorporated the DNA into their genome. These are further checked to ensure the DNA is expressed as a functional TCR, using standard immunological techniques (staining with antibodies, T cell proliferation to antigen etc.). These A1(M) transgenic mice are then crossed with RAG-1$^{-/-}$ deficient mice (Alt et al. 1992 and Mombaerts et al. 1992), and the offspring are backcrossed onto the A1(M) parents for a

number of generations, selecting those that carry both the TCR and RAG-$1^{-/-}$ genes.

The A1 TCR DNA can be made in a number of ways; including the following:

1) By cloning the TCR alpha and beta chain mRNAs as cDNA (standard method) from an original A1 $CD4^{+}$ T cell clone such as the A1 $CD4^{+}$ anti-H-Y T cell clone.

2) By cloning the TCR alpha and beta chain mRNAs as cDNA (standard method) from the A1(M) or A1(M)xRAG-$1^{-/-}$ female mice or $CD4^{+}$ anti-H-Y T cell clones derived from them.

3) By using the DNA sequence information to sythesize the TCR alpha and beta chain genes. This can simply be performed by making a series of overlapping oligonucleotides of convenient length (eg. 30-50 bases each) such that the entire sequence on both strands is covered. Simple annealing and ligation then generates the full length, double stranded DNA for each gene. This method requires only suitable TCR sequence information (for example the sequence in figure 1) to generate the A1(M) mice, using techniques well known in the art of making transgenic mice.

4) By using the DNA sequence information to mutate (standard methods of site-directed mutagenesis) other (related) TCR alpha and beta chain genes to a known desired sequence (either the entire sequence or just the complementarity determining regions (CDRs) that define the antigen binding site) such as the TCR gene sequences shown in figure 1.

Once the TCR alpha and beta chain genes have either been cloned or synthesized they are ligated into an appropriate expression vector or cassette that allows them to be expressed in T cells. The CD2

minigene cassette system described in Zhumabekov et al. 1995 *J. Immunol. Methods* 185(1):133-40 is a suitable expression system.

Legends to Figures

Figure 1 - A1 TCR α and β chains and nucleic acid sequences encoding them.

Figure 2 - Female A1(M)xRAG-1$^{-/-}$ mice show CD4 dependent, specific rejection of male skin

Female A1(M)xRAG-1$^{-/-}$ mice were grafted with male and female CBA/Ca skin in the same graft bed. Survival plots are shown for the male skin that rejected with a median survival time (MST) of 14 days (●; n=5) compared to the accompanying female grafts that survived beyond day 30 (○ ; n=5). The P value for statistical significance was <0.003 (LogRank method). Also shown is the survival of male skin on similarly grafted A1(M)xRAG-1$^{-/-}$ female mice treated with 5 x 1mg non-depeting CD4 antibody (■ ; n=5; MST >30 days).

Figure 3 - Phenotypic and functional immunofluorescent analysis of rejecting A1(M)xRAG$^{-/-}$ mice

Two female A1(M)xRAG-1$^{-/-}$ mice, that had rejected two sequential male skin grafts were given a third graft, and their spleen cells stained for a number of surface and intracytoplasmic antigens. Representative examples of four colour immunofluorescent analysis from one of the mice are shown. All samples were live gated on forward and side scatters, and the dot plot in the upper left panel shows CD4-PE versus CD8α-QR staining of the CD3-FITC positive lymphocytes. The upper right panel shows that there were no B cells expressing surface Ig in the A1(M)xRAG-1$^{-/-}$ (filled histogram) compared to an A1(M) control (broken

line histogram). The centre left panel shows the staining for CD44-QR of A1(M)xRAG-1$^{-/-}$ lymphocytes, that was used as the basis for gating the remaining anti-cytokine stains (rat IgG1 anti-IFN-γ, centre right; rat IgG1 anti-IL-2, lower left; rat IgG1 anti-IL-4, lower right), where the CD44^{+} cells are shown as filled histograms, the CD44^{-} cells as open histograms, and the negative control histogram (based on the background staining of isotype matched, rat IgG1 anti-IL4-FITC in normal mice) is shown with a broken line.

CLAIMS

1. A genetically modified non-human mammal having a population of CD4 positive T cells specific for one or a limited number of selected antigens, including at least one transplantation antigen, the mammal being capable of an immune response against the transplantation antigen and of being tolerised to the transplantation antigen.

2. The genetically modified mammal according to claim 1, which is capable of rejecting a tissue transplant containing the transplantation antigen and the other selected antigens.

3. The genetically modified mammal according to claim 1 or claim 2, having stably integrated into its genome T cell receptor genes which encode a T cell receptor specific for the transplantation antigen.

4. The genetically modified mammal according to any one of claims 1 to 3, which lacks a normal population of CD8 positive T cells or B cells or both.

5. The genetically modified mammal according to claim 4, having no functional CD8 positive T cells or B cells.

6. The genetically modified mammal according to any one of claims 1 to 5, wherein there is a deficiency in lymphocyte receptor recombination such that no T cell or B cell receptors are expressed other than the T cell receptor for the transplantation antigen and if present, T cell receptors for the limited number of other selected antigens.

7. The genetically modified mammal according to any one of claims 1 to 6, wherein a gene involved in lymphocyte receptor recombination is inactivated or deleted.

8. The genetically modified mammal according to claim 7, wherein one or both copies of the RAG-1 and/or RAG-2 gene are inactivated or deleted.

9. The genetically modified mammal according to any one of claims 1 to 8, wherein the transplantation antigen is a male or female transplantation antigen.

10. The genetically modified mammal according to claim 9, wherein the transplantation antigen is the male transplantation antigen H-Y.

11. The genetically modified mammal according to claim 10, wherein the T cell receptor specific for H-Y is encoded by the nucleic acid sequences shown in figure 1.

12. The genetically modified mammal according to any one of claims 1 to 11, for use in studying tolerance.

13. The genetically modified mammal according to any one of claims 1 to 12, for use in drug screening.

14. A method of screening for biologically active compounds which method comprises administering the compounds to a genetically modified non-human mammal according to any one of claims 1 to 13 and observing the effect of the compounds on the ability of the mammal to reject or maintain a transplant containing the transplantation antigen.

15. The method according to claim 14, comprising the further step of employing a biologically active compound identified by the method, as a tolerance enhancing or inducing or suppressing agent.

16. A method which comprises inducing or enhancing or suppressing tolerance by means of a biologically active compound identified by the screening method according to claim 14.

17. A method of investigating immunological changes in tolerance or rejection, which method comprises applying a suitable tissue transplant to a mammal according to any one of claims 1 to 13 under conditions to promote tolerance or rejection, and monitoring changes in one or more immunological indicators such as cytokine levels or T cell activation markers.

18. A method of studying transplantation tolerance or rejection, which method comprises applying a suitable tissue transplant to an animal according to any one of claims 1 to 13 under conditions to promote tolerance to or rejection of the transplant, and assessing biological changes in the animal.

19. A method of studying immunological tolerance in pregancy, which method comprises providing a pregnant female animal according to any one of claims 1 to 13, said female animal carrying at least one foetus displaying the transplantation antigen, and monitoring changes in one or more immunological indicators such as cytokine levels or T cell activation markers.

Figure 1

<u>A1 TCR alpha chain</u> [SEQ ID NO: 5 and 6]

MKRLLCSLLGLLCTQVCWVKGQQVQQSPASLVLQEGENAELQCNFSTSLNSMQWFYQRPEGSLVSL
FYNPSGTKQSGRLTSTTVIKERRSSLHISSSQITDSGTYLCADWTGNTRKLIFGLGTTLQVQPDIQ
NPEPAVYQLKDPRSQDSTLCLFTDFDSQINVPKTMESGTFITDKTVLDMKAMDSKSNGAIAWSNQT
SFTCQDIFKETNATYPSSDVPCDATLTEKSFETDMNLNFQNLSVMGLRILLLKVAGFNLLMTLRLW
SS

```
acaagcacca tgaagaggct gctgtgctct ctgctggggc ttctgtgcac ccaggtttgc     60
tgggtgaaag gacagcaagt gcagcagagc cccgcgtcct tggttctgca ggaggggag     120
aatgcagagc tgcagtgtaa cttttccaca tctttgaaca gtatgcagtg gttttaccaa    180
cgtcctgagg gaagtctcgt cagcctgttc tacaatcctt ctgggacaaa gcagagtggg    240
agactgacat ccacaacagt catcaaagaa cgtcgcagct ctttgcacat ttcctcctcc    300
cagatcacag actcaggcac ttatctctgt gccgattgga caggcaatac tagaaaactc    360
atctttgggc tggggacaac tttacaagtg caaccagaca tccagaaccc agaacctgct    420
gtgtaccagt taaaagatcc tcggtctcag gacagcaccc tctgcctgtt caccgacttt    480
gactcccaaa tcaatgtgcc gaaaaccatg gaatctggaa cgttcatcac tgacaaaact    540
gtgctggaca tgaaagctat ggattccaag agcaatgggg ccattgcctg gagcaaccag    600
acaagcttca cctgccaaga tatcttcaaa gagaccaacg ccacctaccc cagttcagac    660
gttccctgtg atgccacgtt gaccgagaaa agctttgaaa cagatatgaa cctaaacttt    720
caaaacctgt cagttatggg actccgcatc ctcctgctga aagtagcggg atttaacctg    780
ctcatgacgc tgaggctgtg gtccagttga ggtct                                815
```

<u>A1 TCR beta chain</u> [SEQ ID NO: 7 and 8]

MSNTAFPDPAWNTTLLSWVALFLLGTKHMEAAVTQSPRNKVAVTGGKVTLSCNQTNNHNNMYWYRQ
DTGHGLRLIHYSYGAGSTEKGDIPDGYKASRPSQENFSLILELATPSQTSVYFCASGDSGLGSETL
YFGSGTRLTVLEDLRNVTPPKVSLFEPSKAEIANKQKATLVCLARGFFPDHVELSWWVNGKEVHSG
VSTDPQAYKESNYSYCLSSRLRVSATFWHNPRNHFRCQVQFHGLSEEDKWPEGSPKPVHRTSVQRP
GAEQCGITSSYHQGVLSATILYEILLGKATLYAVLVSGLVLMAMVKKKNS

agaggaagca tgtctaacac tgccttccct gaccccgcct ggaacaccac cctgctatct 60
tgggttgctc tctttctcct gggaacaaaa cacatggagg ctgcagtcac ccaaagccca 120
agaaacaagg tggcagtaac aggaggaaag gtgacattga gctgtaatca gactaataac 180
cacaacaaca tgtactggta tcggcaggac acggggcatg ggctgaggct gatccattat 240
tcatatggtg ctggcagcac tgagaaagga gatatccctg atggatacaa ggcctccaga 300
ccaagccaag agaacttctc cctcattctg gagttggcta ccccctctca gacatcagtg 360
tacttctgtg ccagcggtga ttcgggactg gggtcagaaa cgctgtattt tggctcagga 420
accagactga ctgttctcga ggatctgaga aatgtgactc cacccaaggt ctccttgttt 480
gagccatcaa aagcagagat tgcaaacaaa caaaaggcta ccctcgtgtg cttggccagg 540
ggcttcttcc ctgaccacgt ggagctgagc tggtgggtga atggcaagga ggtccacagt 600
ggggtcagca cggaccctca ggcctacaag gagagcaatt atagctactg cctgagcagc 660
cgcctgaggg tctctgctac cttctggcac aatcctcgaa accacttccg ctgccaagtg 720
cagttccatg ggctttcaga ggaggacaag tggccagagg gctcacccaa acctgtacac 780
agaacatcag tgcagaggcc tggggccgag cagtgtggaa tcacttcatc ctatcatcag 840
ggggttctgt ctgcaaccat cctctatgag atcctactgg ggaaggccac cctatatgct 900
gtgctggtca gtggcctagt gctgatggcc atggtcaaga aaaaaaattc ctga 954

Figure 2

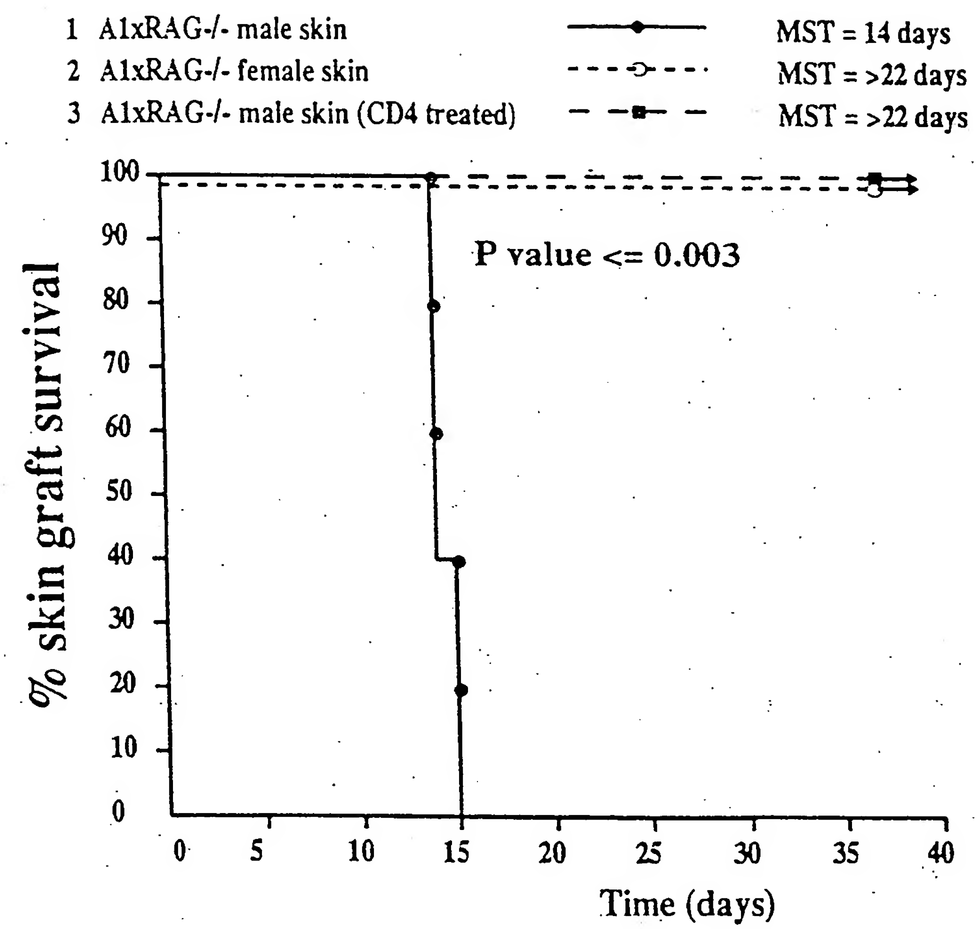
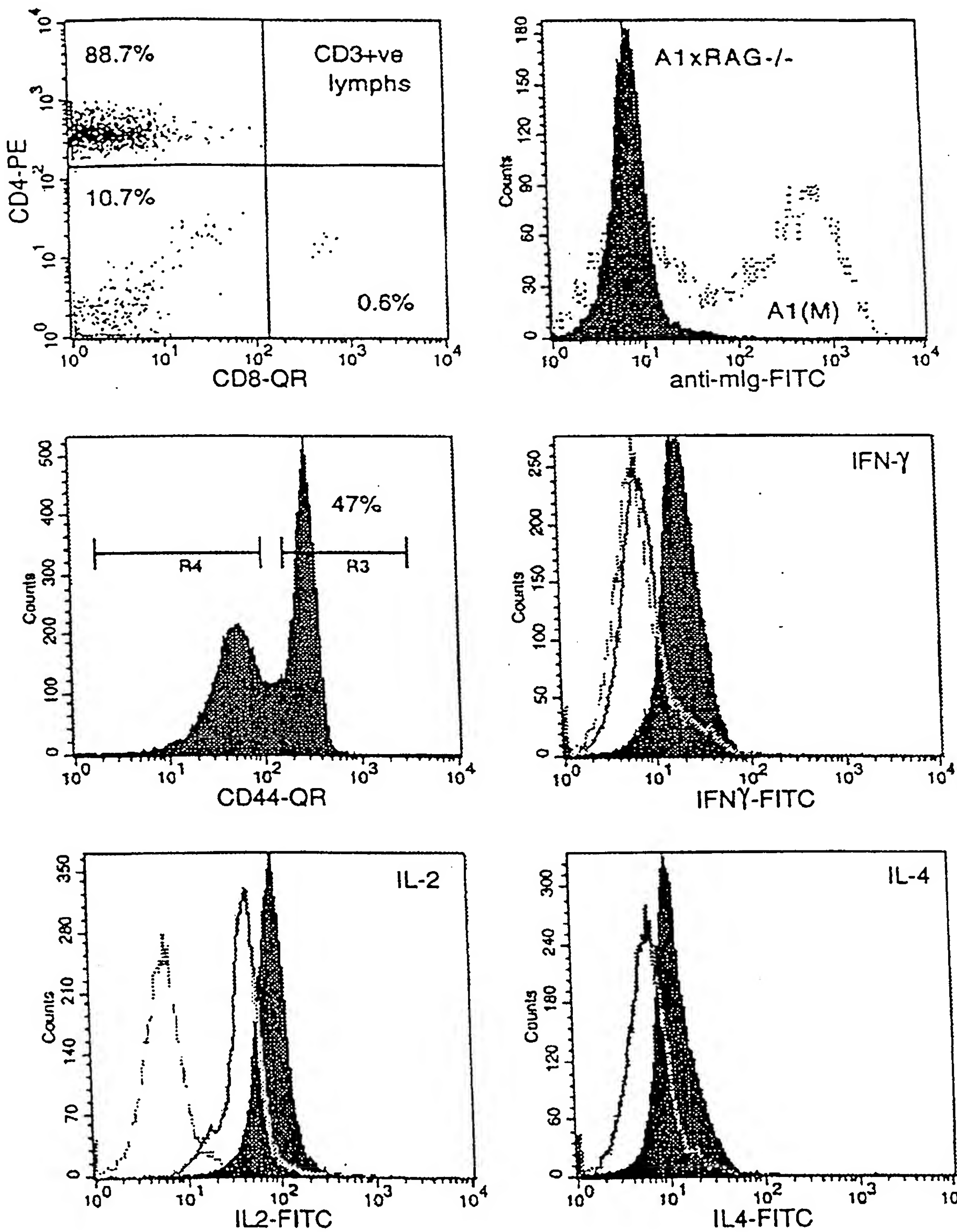

1 A1xRAG-/- male skin
MST = 14 days
2 A1xRAG-/- female skin
MST = >22 days
3 A1xRAG-/- male skin (CD4 treated)
MST = >22 days
P value <= 0.003
% skin graft survival
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
Time (days)

Figure 3


88.7%
CD3+ve lymphs
10.7%
0.6%
CD4-PE
CD8-QR
A1xRAG-/-
A1(M)
Counts
anti-mIg-FITC
47%
R4
R3
Counts
CD44-QR
IFN-γ
Counts
IFNγ-FITC
IL-2
Counts
IL2-FITC
IL-4
Counts
IL4-FITC

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: ISIS INNOVATION LIMITED
(B) STREET: 2 SOUTH PARKS ROAD
(C) CITY: OXFORD
(D) STATE: OXON
(E) COUNTRY: UNITED KINGDOM
(F) POSTAL CODE (ZIP): OX1 3UB

(ii) TITLE OF INVENTION: TOLERANCE

(iii) NUMBER OF SEQUENCES: 8

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: GB 9720888.8

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCGAATCACA AGCACCATGA AGAGGCTG 28

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GCGAATTCCA GACCTCAACT GGACCACAG 29

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCGAATTCAG AGGAAGCATG TCTAACACT 29

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCGAATTCAG GATGCATAAA AGTTTGTCTC AGG 33

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 815 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Mus musculus

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:10..810
(D) OTHER INFORMATION:/codon_start= 1
/product= "A1 TCR alpha chain"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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ACAAGCACC ATG AAG AGG CTG CTG TGC TCT CTG CTG GGG CTT CTG TGC     48
          Met Lys Arg Leu Leu Cys Ser Leu Leu Gly Leu Leu Cys
           1               5                  10
```

```
ACC CAG GTT TGC TGG GTG AAA GGA CAG CAA GTG CAG CAG AGC CCC GCG      96
Thr Gln Val Cys Trp Val Lys Gly Gln Gln Val Gln Gln Ser Pro Ala
 15                  20                  25

TCC TTG GTT CTG CAG GAG GGG GAG AAT GCA GAG CTG CAG TGT AAC TTT     144
Ser Leu Val Leu Gln Glu Gly Glu Asn Ala Glu Leu Gln Cys Asn Phe
 30                  35                  40                  45

TCC ACA TCT TTG AAC AGT ATG CAG TGG TTT TAC CAA CGT CCT GAG GGA     192
Ser Thr Ser Leu Asn Ser Met Gln Trp Phe Tyr Gln Arg Pro Glu Gly
                 50                  55                  60

AGT CTC GTC AGC CTG TTC TAC AAT CCT TCT GGG ACA AAG CAG AGT GGG     240
Ser Leu Val Ser Leu Phe Tyr Asn Pro Ser Gly Thr Lys Gln Ser Gly
             65                  70                  75

AGA CTG ACA TCC ACA ACA GTC ATC AAA GAA CGT CGC AGC TCT TTG CAC     288
Arg Leu Thr Ser Thr Thr Val Ile Lys Glu Arg Arg Ser Ser Leu His
         80                  85                  90

ATT TCC TCC TCC CAG ATC ACA GAC TCA GGC ACT TAT CTC TGT GCC GAT     336
Ile Ser Ser Ser Gln Ile Thr Asp Ser Gly Thr Tyr Leu Cys Ala Asp
     95                 100                 105

TGG ACA GGC AAT ACT AGA AAA CTC ATC TTT GGG CTG GGG ACA ACT TTA     384
Trp Thr Gly Asn Thr Arg Lys Leu Ile Phe Gly Leu Gly Thr Thr Leu
110                 115                 120                 125

CAA GTG CAA CCA GAC ATC CAG AAC CCA GAA CCT GCT GTG TAC CAG TTA     432
Gln Val Gln Pro Asp Ile Gln Asn Pro Glu Pro Ala Val Tyr Gln Leu
                130                 135                 140

AAA GAT CCT CGG TCT CAG GAC AGC ACC CTC TGC CTG TTC ACC GAC TTT     480
Lys Asp Pro Arg Ser Gln Asp Ser Thr Leu Cys Leu Phe Thr Asp Phe
            145                 150                 155

GAC TCC CAA ATC AAT GTG CCG AAA ACC ATG GAA TCT GGA ACG TTC ATC     528
Asp Ser Gln Ile Asn Val Pro Lys Thr Met Glu Ser Gly Thr Phe Ile
        160                 165                 170

ACT GAC AAA ACT GTG CTG GAC ATG AAA GCT ATG GAT TCC AAG AGC AAT     576
Thr Asp Lys Thr Val Leu Asp Met Lys Ala Met Asp Ser Lys Ser Asn
    175                 180                 185

GGG GCC ATT GCC TGG AGC AAC CAG ACA AGC TTC ACC TGC CAA GAT ATC     624
Gly Ala Ile Ala Trp Ser Asn Gln Thr Ser Phe Thr Cys Gln Asp Ile
190                 195                 200                 205

TTC AAA GAG ACC AAC GCC ACC TAC CCC AGT TCA GAC GTT CCC TGT GAT     672
Phe Lys Glu Thr Asn Ala Thr Tyr Pro Ser Ser Asp Val Pro Cys Asp
                210                 215                 220

GCC ACG TTG ACC GAG AAA AGC TTT GAA ACA GAT ATG AAC CTA AAC TTT     720
Ala Thr Leu Thr Glu Lys Ser Phe Glu Thr Asp Met Asn Leu Asn Phe
            225                 230                 235
```

```
CAA AAC CTG TCA GTT ATG GGA CTC CGC ATC CTC CTG CTG AAA GTA GCG    768
Gln Asn Leu Ser Val Met Gly Leu Arg Ile Leu Leu Leu Lys Val Ala
        240                 245                 250

GGA TTT AAC CTG CTC ATG ACG CTG AGG CTG TGG TCC AGT TGA            810
Gly Phe Asn Leu Leu Met Thr Leu Arg Leu Trp Ser Ser  *
    255                 260                 265

GGTCT                                                              815
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(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 267 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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Met Lys Arg Leu Leu Cys Ser Leu Leu Gly Leu Leu Cys Thr Gln Val
  1               5                  10                  15

Cys Trp Val Lys Gly Gln Gln Val Gln Gln Ser Pro Ala Ser Leu Val
             20                  25                  30

Leu Gln Glu Gly Glu Asn Ala Glu Leu Gln Cys Asn Phe Ser Thr Ser
         35                  40                  45

Leu Asn Ser Met Gln Trp Phe Tyr Gln Arg Pro Glu Gly Ser Leu Val
     50                  55                  60

Ser Leu Phe Tyr Asn Pro Ser Gly Thr Lys Gln Ser Gly Arg Leu Thr
 65                  70                  75                  80

Ser Thr Thr Val Ile Lys Glu Arg Arg Ser Ser Leu His Ile Ser Ser
                 85                  90                  95

Ser Gln Ile Thr Asp Ser Gly Thr Tyr Leu Cys Ala Asp Trp Thr Gly
            100                 105                 110

Asn Thr Arg Lys Leu Ile Phe Gly Leu Gly Thr Thr Leu Gln Val Gln
        115                 120                 125

Pro Asp Ile Gln Asn Pro Glu Pro Ala Val Tyr Gln Leu Lys Asp Pro
    130                 135                 140

Arg Ser Gln Asp Ser Thr Leu Cys Leu Phe Thr Asp Phe Asp Ser Gln
145                 150                 155                 160

Ile Asn Val Pro Lys Thr Met Glu Ser Gly Thr Phe Ile Thr Asp Lys
                165                 170                 175

Thr Val Leu Asp Met Lys Ala Met Asp Ser Lys Ser Asn Gly Ala Ile
            180                 185                 190
```

```
Ala Trp Ser Asn Gln Thr Ser Phe Thr Cys Gln Asp Ile Phe Lys Glu
        195                 200                 205

Thr Asn Ala Thr Tyr Pro Ser Ser Asp Val Pro Cys Asp Ala Thr Leu
    210                 215                 220

Thr Glu Lys Ser Phe Glu Thr Asp Met Asn Leu Asn Phe Gln Asn Leu
225                 230                 235                 240

Ser Val Met Gly Leu Arg Ile Leu Leu Leu Lys Val Ala Gly Phe Asn
                245                 250                 255

Leu Leu Met Thr Leu Arg Leu Trp Ser Ser  *
            260                 265
```

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 954 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Mus musculus

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:10..954
(D) OTHER INFORMATION:/codon_start= 1
/product= "A1 TCR beta chain"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

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AGAGGAAGC ATG TCT AAC ACT GCC TTC CCT GAC CCC GCC TGG AAC ACC      48
          Met Ser Asn Thr Ala Phe Pro Asp Pro Ala Trp Asn Thr
                  270                 275                 280

ACC CTG CTA TCT TGG GTT GCT CTC TTT CTC CTG GGA ACA AAA CAC ATG    96
Thr Leu Leu Ser Trp Val Ala Leu Phe Leu Leu Gly Thr Lys His Met
                285                 290                 295

GAG GCT GCA GTC ACC CAA AGC CCA AGA AAC AAG GTG GCA GTA ACA GGA   144
Glu Ala Ala Val Thr Gln Ser Pro Arg Asn Lys Val Ala Val Thr Gly
            300                 305                 310

GGA AAG GTG ACA TTG AGC TGT AAT CAG ACT AAT AAC CAC AAC AAC ATG   192
Gly Lys Val Thr Leu Ser Cys Asn Gln Thr Asn Asn His Asn Asn Met
        315                 320                 325

TAC TGG TAT CGG CAG GAC ACG GGG CAT GGG CTG AGG CTG ATC CAT TAT   240
Tyr Trp Tyr Arg Gln Asp Thr Gly His Gly Leu Arg Leu Ile His Tyr
    330                 335                 340
```

```
TCA TAT GGT GCT GGC AGC ACT GAG AAA GGA GAT ATC CCT GAT GGA TAC    288
Ser Tyr Gly Ala Gly Ser Thr Glu Lys Gly Asp Ile Pro Asp Gly Tyr
345                 350                 355                 360

AAG GCC TCC AGA CCA AGC CAA GAG AAC TTC TCC CTC ATT CTG GAG TTG    336
Lys Ala Ser Arg Pro Ser Gln Glu Asn Phe Ser Leu Ile Leu Glu Leu
                365                 370                 375

GCT ACC CCC TCT CAG ACA TCA GTG TAC TTC TGT GCC AGC GGT GAT TCG    384
Ala Thr Pro Ser Gln Thr Ser Val Tyr Phe Cys Ala Ser Gly Asp Ser
            380                 385                 390

GGA CTG GGG TCA GAA ACG CTG TAT TTT GGC TCA GGA ACC AGA CTG ACT    432
Gly Leu Gly Ser Glu Thr Leu Tyr Phe Gly Ser Gly Thr Arg Leu Thr
        395                 400                 405

GTT CTC GAG GAT CTG AGA AAT GTG ACT CCA CCC AAG GTC TCC TTG TTT    480
Val Leu Glu Asp Leu Arg Asn Val Thr Pro Pro Lys Val Ser Leu Phe
    410                 415                 420

GAG CCA TCA AAA GCA GAG ATT GCA AAC AAA CAA AAG GCT ACC CTC GTG    528
Glu Pro Ser Lys Ala Glu Ile Ala Asn Lys Gln Lys Ala Thr Leu Val
425                 430                 435                 440

TGC TTG GCC AGG GGC TTC TTC CCT GAC CAC GTG GAG CTG AGC TGG TGG    576
Cys Leu Ala Arg Gly Phe Phe Pro Asp His Val Glu Leu Ser Trp Trp
                445                 450                 455

GTG AAT GGC AAG GAG GTC CAC AGT GGG GTC AGC ACG GAC CCT CAG GCC    624
Val Asn Gly Lys Glu Val His Ser Gly Val Ser Thr Asp Pro Gln Ala
            460                 465                 470

TAC AAG GAG AGC AAT TAT AGC TAC TGC CTG AGC AGC CGC CTG AGG GTC    672
Tyr Lys Glu Ser Asn Tyr Ser Tyr Cys Leu Ser Ser Arg Leu Arg Val
        475                 480                 485

TCT GCT ACC TTC TGG CAC AAT CCT CGA AAC CAC TTC CGC TGC CAA GTG    720
Ser Ala Thr Phe Trp His Asn Pro Arg Asn His Phe Arg Cys Gln Val
    490                 495                 500

CAG TTC CAT GGG CTT TCA GAG GAG GAC AAG TGG CCA GAG GGC TCA CCC    768
Gln Phe His Gly Leu Ser Glu Glu Asp Lys Trp Pro Glu Gly Ser Pro
505                 510                 515                 520

AAA CCT GTA CAC AGA ACA TCA GTG CAG AGG CCT GGG GCC GAG CAG TGT    816
Lys Pro Val His Arg Thr Ser Val Gln Arg Pro Gly Ala Glu Gln Cys
                525                 530                 535

GGA ATC ACT TCA TCC TAT CAT CAG GGG GTT CTG TCT GCA ACC ATC CTC    864
Gly Ile Thr Ser Ser Tyr His Gln Gly Val Leu Ser Ala Thr Ile Leu
            540                 545                 550

TAT GAG ATC CTA CTG GGG AAG GCC ACC CTA TAT GCT GTG CTG GTC AGT    912
Tyr Glu Ile Leu Leu Gly Lys Ala Thr Leu Tyr Ala Val Leu Val Ser
        555                 560                 565
```

```
GGC CTA GTG CTG ATG GCC ATG GTC AAG AAA AAA AAT TCC TGA          954
Gly Leu Val Leu Met Ala Met Val Lys Lys Lys Asn Ser  *
    570                 575                 580
```

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 315 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

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Met Ser Asn Thr Ala Phe Pro Asp Pro Ala Trp Asn Thr Thr Leu Leu
  1               5                  10                  15

Ser Trp Val Ala Leu Phe Leu Leu Gly Thr Lys His Met Glu Ala Ala
            20                  25                  30

Val Thr Gln Ser Pro Arg Asn Lys Val Ala Val Thr Gly Gly Lys Val
        35                  40                  45

Thr Leu Ser Cys Asn Gln Thr Asn Asn His Asn Asn Met Tyr Trp Tyr
    50                  55                  60

Arg Gln Asp Thr Gly His Gly Leu Arg Leu Ile His Tyr Ser Tyr Gly
 65                  70                  75                  80

Ala Gly Ser Thr Glu Lys Gly Asp Ile Pro Asp Gly Tyr Lys Ala Ser
                 85                  90                  95

Arg Pro Ser Gln Glu Asn Phe Ser Leu Ile Leu Glu Leu Ala Thr Pro
            100                 105                 110

Ser Gln Thr Ser Val Tyr Phe Cys Ala Ser Gly Asp Ser Gly Leu Gly
        115                 120                 125

Ser Glu Thr Leu Tyr Phe Gly Ser Gly Thr Arg Leu Thr Val Leu Glu
    130                 135                 140

Asp Leu Arg Asn Val Thr Pro Pro Lys Val Ser Leu Phe Glu Pro Ser
145                 150                 155                 160

Lys Ala Glu Ile Ala Asn Lys Gln Lys Ala Thr Leu Val Cys Leu Ala
                165                 170                 175

Arg Gly Phe Phe Pro Asp His Val Glu Leu Ser Trp Trp Val Asn Gly
            180                 185                 190

Lys Glu Val His Ser Gly Val Ser Thr Asp Pro Gln Ala Tyr Lys Glu
        195                 200                 205

Ser Asn Tyr Ser Tyr Cys Leu Ser Ser Arg Leu Arg Val Ser Ala Thr
    210                 215                 220
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Phe Trp His Asn Pro Arg Asn His Phe Arg Cys Gln Val Gln Phe His
225 230 235 240

Gly Leu Ser Glu Glu Asp Lys Trp Pro Glu Gly Ser Pro Lys Pro Val
245 250 255

His Arg Thr Ser Val Gln Arg Pro Gly Ala Glu Gln Cys Gly Ile Thr
260 265 270

Ser Ser Tyr His Gln Gly Val Leu Ser Ala Thr Ile Leu Tyr Glu Ile
275 280 285

Leu Leu Gly Lys Ala Thr Leu Tyr Ala Val Leu Val Ser Gly Leu Val
290 295 300

Leu Met Ala Met Val Lys Lys Lys Asn Ser *
305 310 315

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/02965

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/00 A01K67/027 C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCULLY, R. ET AL.: "A role for Th2 cytokines in the suppression of CD8+ T cell-mediated graft rejection" EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 27, no. 7, July 1997, pages 1663-1670, XP002091475 MONTREUIL FR see page 1663, column 2, paragraph 2 ---	1,3
X	HÄMMERLING, G.J. ET AL.: "Peripheral tolerance as a multi-step mechanism" IMMUNOLOGICAL REVIEWS, vol. 133, 1993, pages 93-104, XP002091476 cited in the application see the whole document ---	1,3,12, 17,18

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 January 1999	18/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Chambonnet, F

3

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	KOUSKOFF V ET AL: "Cassette vectors directing expression of T cell receptor genes in transgenic mice" JOURNAL OF IMMUNOLOGICAL METHODS, vol. 180, no. 2, 27 March 1995, page 273-280 XP004021050 see page 276, column 2, line 1 ---	1,9,10
A	WO 90 06359 A (GENPHARM INT) 14 June 1990 see the whole document ---	1-7
A	WO 95 32285 A (INST NAT SANTE RECH MED ;UNIV PASTEUR (FR); CENTRE NAT RECH SCIENT) 30 November 1995 see the whole document ---	1
A	WO 97 08303 A (UNIV TECHNOLOGIES INT) 6 March 1997 see the whole document ---	1
A	MARSHALL, S. E. ET AL.: "Tolerance and suppression in a primed immune system" TRANSPLANTATION, vol. 62, no. 11, 15 December 1996, pages 1614-1621, XP002091477 see the whole document ---	1
A	COBBOLD, S.P. ET AL.: "Mechanisms of peripheral tolerance and suppression induced by monoclonal antibodies to CD4 and CD8" IMMUNOLOGICAL REVIEWS, vol. 0, no. 149, 1996, pages 5-33, XP002091478 cited in the application see the whole document ---	1
P,X	ZELENIKA, D. ET AL.: "Rejection of H-Y disparate skin grafts by monospecific CD4+ T helper 1 (Th1) and T helper 2 (Th2) cells : no requirement for CD8+T cells or B cells" JOURNAL OF IMMUNOLOGY, vol. 161, no. 4, 15 August 1998, pages 1868-1874, XP002091479 see the whole document ---	1
A,P	WO 97 35991 A (UNIV JOHNS HOPKINS ;SCHNECK JONATHAN P (US); HERRIN SEAN O (US)) 2 October 1997 see the whole document -----	1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 98/02965

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Further Information sheet enclosed.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

As far as claim 16 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition.

Although claims 17 and 18 are directed to surgical methods applied to the human/animal body, the search has been carried out and based on the alleged effects of the composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 98/02965

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9006359 A	14-06-1990	US 5175384 A	29-12-1992
		AU 4755790 A	26-06-1990
		CA 2004477 A	05-06-1990
		US 5591669 A	07-01-1997
		US 5434340 A	18-07-1995
WO 9532285 A	30-11-1995	US 5675060 A	07-10-1997
		CA 2189740 A	30-11-1995
		EP 0759985 A	05-03-1997
		JP 10504701 T	12-05-1998
WO 9708303 A	06-03-1997	AU 6730096 A	19-03-1997
WO 9735991 A	02-10-1997	AU 2422497 A	17-10-1997
		EP 0889964 A	13-01-1999